

## Longitudinal Study of Immune Response in Human Chagas' Disease

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Immune response, clinical status, and reactivity to heart tissue were studied longitudinally for 1 year in 42 patients with Chagas' disease (South American trypanosomiasis). The patients were divided into two groups. Group 1 was composed of patients with chagasic infection with no evidence of heart disease. Group 2 patients had chagasic infection and cardiomyopathy. Humoral immune response to *Trypanosoma cruzi* was measured serologically, and cell-mediated immune responses to *T. cruzi* and rat heart antigens were evaluated by lymphoblastogenesis. Parasitemia was detected by xenodiagnosis. Serological tests for anti-*T. cruzi* antibodies were positive in all patients of both groups, and the titers were significantly higher in group 2. A change of titer during the study period was more frequently associated with a positive xenodiagnosis in both groups. Lymphoblastogenesis in response to *T. cruzi* antigen was positive at least once in all patients of both groups. When rat heart antigen was used, 44.4% of the patients in group 1 and 40.0% of those in group 2 were positive on at least one occasion. Xenodiagnosis revealed that 20% of the patients in group 1 and 50% of those in group 2 ( $P = 0.01$ ) had detectable circulating parasites during the course of the study. Positive xenodiagnosis was associated with lower lymphoblastogenic responses to *T. cruzi* in group 1 patients, suggesting the presence of a regulatory or modulatory mechanism which is lost in patients with chagasic cardiomyopathy. No relationship between positive xenodiagnosis and positive lymphoblastogenesis in response to heart antigen could be established. In addition, no correlation was found between clinical heart disease and reactivity to rat heart tissue.

Both humoral and cell-mediated immune responses to *Trypanosoma cruzi* antigen are present during the chronic phase of Chagas' disease in humans (10, 11, 13, 16, 20). However, despite seemingly adequate immune responses, the parasites persist in the blood and tissues, albeit at low levels that require culture or xenodiagnosis for detection. It is during the chronic phase that some individuals develop progressive cardiomyopathy, the main feature of the disease in Venezuela. Because the extent of chronic cardiomyopathy is out of proportion to the few persisting organisms, there has been much speculation that autosensitization to a heart antigen is involved in its pathogenesis. In a test of this hypothesis (12), we found that the prevalence of lymphoblastogenic response to heterologous rat heart antigen in patients with chagasic cardiomyopathy (26.8%) did not differ from that in infected individuals without cardiomyopathy (25.0%). The case-control design used in this initial study failed to examine potentially important fluctuations in anti-heart activity occurring over time and their relation to changes in the clinical status of patients that could be related to pathogenesis. To examine this possibility, we prospectively examined lymphoblastogenic response to *T. cruzi* antigen and to heterologous rat heart antigen in 15 patients with Chagas' disease and cardiomyopathy and 27 infected individuals without cardiomyopathy. In addition, we studied the relationships among in vitro lymphoblastogenic response to the presence of parasitemia, the level of serum antibodies to *T. cruzi*, and the clinical status of patients.

### MATERIALS AND METHODS

**Patient groups.** Participants in the study were selected from patients visiting the cardiology clinic for Chagas' disease and cardiomyopathy at the J. M. Vargas Hospital in Caracas. A total of 42 patients with a positive complement fixation test (CFT) for antibody to *T. cruzi* agreed to participate in the longitudinal study. The same clinical and electrocardiographic criteria used to classify patients in our previous study (11) were used to assign participants to two groups. The 27 patients assigned to group 1 (19 males and 8 females; mean age, 37 years; range, 21 to 53 years) were seropositive but had no evidence of heart disease. The 15 patients assigned to group 2 (10 males and 5 females; mean age, 42.9 years; range, 27 to 62 years) had electrocardiographic abnormalities characteristic of either complete right bundle branch block plus left anterior or posterior fascicular block or complete atrioventricular block with a wide QRS complex.

Once classified, each patient was reexamined at least three times over a 1-year period with a minimal interval of 2 months between tests. Each examination consisted of clinical and electrocardiographic evaluation, in vitro lymphoblastogenesis test, serological tests, and xenodiagnosis. The physical examination and electrocardiography were performed on the day before the laboratory test. If intercurrent illness was detected, the laboratory tests were delayed until the condition resolved.

**Serological tests.** All sera were stored at  $-20^{\circ}\text{C}$  and examined after completion of the study. To avoid test variabilities, the serial serum samples from a given patient were

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TABLE 1. Distribution of antibody titers

Antibody titer	No. of positive tests (% of total) in group:					
	CFT <sup>a</sup>		Hemagglutination <sup>b</sup>		Immunofluorescence <sup>b</sup>	
	1	2	1	2	1	2
1/2						
1/4						
1/8	10 (8)	4 (6)				
1/16	32 (26)	6 (9)				
1/32	29 (23)	18 (28)	8 (6)	2 (3)	1 (0.6)	
1/64 <sup>c</sup>	54 (43)	36 (56) <sup>d</sup>	28 (21)	7 (10) <sup>d</sup>	22 (16)	7 (10)
1/128			39 (30)	13 (19)	58 (43)	24 (35)
1/256			28 (21)	17 (25)	25 (19)	18 (26)
1/512			28 (21)	29 (42) <sup>d</sup>	28 (21)	19 (28) <sup>d</sup>

<sup>a</sup> Diagnostic titer, 1/4.<sup>b</sup> Diagnostic titer, 1/32.<sup>c</sup> For CFT, 1/64 was the highest dilution tested.<sup>d</sup>  $P < 0.05$  when the frequencies of antibody titers in group 1 and 2 were composed by a contingency table.

assayed on the same day with the same lot of *T. cruzi* antigen. For the CFT, we used the Freitas (5) technique and a soluble epimastigote antigen prepared by the method of Maekelt (8). The indirect hemagglutination test was performed by the method of Boyden (1) as modified by Cerisola (4) for Chagas' disease. Indirect immunofluorescence was done by the method of Camargo (2).

**Lymphoblastogenesis assay.** The lymphoblastogenesis assay of Ulrich et al. (21) was used. Briefly, 15 to 20 ml of heparinized blood was allowed to stand for 60 min to sediment the erythrocytes. Adherent cells were removed by incubating the leukocyte-rich plasma in a rectangular glass bottle for 1 h at 37°C. The nonadherent cell population was adjusted to 10<sup>6</sup>/ml by dilution with McCoy medium, the final concentration of autologous plasma always being greater than 10%. For the assay, 1-ml samples of the suspension were cultured in duplicate at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air in the presence or absence of antigens. After 7 days of incubation, the cultures were pulsed for 18 h with 1.0 µCi of [<sup>3</sup>H]thymidine and then harvested. [<sup>3</sup>H]thymidine incorporation was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Results were expressed either as mean net counts per minute (NCPM) or the stimulation index (SI) (mean counts per minute of the antigen-stimulated culture divided by the mean counts per minute of the control culture). SIs equal to or greater than 3.5 were considered indicative of reactivity as reported previously (8, 11–13).

**Antigens for lymphoblastogenesis.** *T. cruzi* antigen was prepared from epimastigotes of the Y strain grown in medium 199 supplemented with 2% fetal calf serum. The

organisms were washed twice with Hanks balanced salt solution and processed by the method indicated below. For antigen preparation A, the suspension was adjusted to 3 × 10<sup>7</sup>/ml and autoclaved for 10 min; 0.05 ml of the preparation was added to the lymphocyte cultures. For antigen preparation B, the suspension was frozen and thawed 10 times and centrifuged at 650 × *g* for 15 min; an equivalent of 75 µg of parasite protein in the supernatant fluid, determined by the modified Lowry method (14), was added to the lymphocyte cultures. For antigen preparation TCS, the sediment of antigen preparation B was suspended in phosphate-buffered saline; an equivalent of 75 µg of parasite protein was added to the lymphocyte cultures.

A heart antigen was prepared from the ventricular mass of young rat hearts. The tissue was minced in Hanks balanced salt solution, frozen and thawed 10 times, and allowed to settle for 15 min. The equivalent of 100 µg of protein in the supernatant fluid was used as an antigen in the lymphocyte cultures.

**Xenodiagnosis.** The Torrealba method (3) of artificial xenodiagnosis was used to detect parasitemia. Briefly, the procedure entails the feeding of heparinized patient blood to 40 *Rhodnius prolixus* fourth-instar nymphs through a membrane. The bugs were examined 30 days later for the presence of *T. cruzi*.

**Statistical evaluation.** A contingency table was constructed to test for between-group differences in antibody titers by the chi-square test. For lymphoblastogenesis results, between-group SI differences were tested by the Student *t* test, and the intergroup differences in NCPM were tested by the Mann-Whitney *U*-rank test.

## RESULTS

**Serology titers and parasitemia.** Analysis of the titers obtained (Table 1) demonstrated a statistically significant ( $P < 0.05$ ) greater frequency of elevated titers among patients with chagasic cardiomyopathy (group 2) than among noncardiomyopathy patients. The variation in serological titers and its relationship with the presence of a positive xenodiagnosis is shown in Table 2. The variation in serological titer was slightly higher in group 2 with CFT and immunofluorescence. When the variation in serological titer was analyzed in each group as a function of a positive xenodiagnosis, it was found that the frequency of serological titer change was higher among patients with a positive xenodiagnosis irrespective of disease status. The prevalence of at least one positive xenodiagnosis during this longitudinal study was significantly ( $P < 0.05$ ) higher in group 2 than in group 1 (Table 3).

**Lymphocyte proliferation.** Mean NCPM and SI with *T. cruzi* and rat heart antigens were not significantly different in the groups studied. All patients reacted to *T. cruzi* antigen with at least one SI value equal to or greater than 3.5, whereas only 44% of group 1 patients and 40% of group 2 patients responded at least once to rat heart antigen. No patient demonstrated persistent reactivity to rat heart anti-

TABLE 2. Patients with changes in serological titer greater than one dilution with respect to xenodiagnosis

Serological test	% of patients with xenodiagnosis in group:					
	1			2		
	Total	Posi- tive	Nega- tive	Total	Posi- tive	Nega- tive
CFT	48	71.5	42.2	60	71.5	42.9
Hemagglutina- tion	50.1	85.7	36.9	35.7	42.9	28.6
Immunofluo- rescence	50	42.9	52.7	64.3	85.7	42.9

TABLE 3. Frequency of positive xenodiagnosis

Group	% of patients with a positive xenodiagnosis	% of patients with at least one positive xenodiagnosis
1	9.1	22.0
2	20.3	50.0 <sup>a</sup>

<sup>a</sup>  $P < 0.05$  compared with group 1.

TABLE 4. SI and NCPM in response to *T. cruzi* antigen with respect to xenodiagnosis

Group (xenodiagnosis)	No. of tests	Mean test results $\pm$ SEM by:	
		SI	NCPM
1			
Positive	41	10.9 $\pm$ 1.4	11,042 $\pm$ 1,076
Negative	96	16.2 $\pm$ 1.5 <sup>a</sup>	17,349 $\pm$ 2,162 <sup>a</sup>
2			
Positive	39	17.23 $\pm$ 3.2	14,216 $\pm$ 2,780
Negative	30	17.10 $\pm$ 2.3	14,044 $\pm$ 3,236

<sup>a</sup>  $P < 0.05$  compared with group 1 total.

gen during our study, and only three patients in group 1 and one patient in group 2 had two consecutive positive lymphocyte proliferation tests in response to rat heart antigen.

When the SI and NCPM in response to *T. cruzi* antigen of patients with a positive xenodiagnosis were compared with those of patients with a negative xenodiagnosis (Table 4), it was found that in group 1 the SI and NCPM were lower in the subgroup with a positive xenodiagnosis; this difference was statistically significant ( $P < 0.05$ ). In contrast, no difference was found in group 2.

**Clinical evaluation.** During the longitudinal study, no change in clinical status could be detected in group 1 patients (mean time of observation, 11.1  $\pm$  2.9 months). In group 2, there was a fluctuation in the clinical status of two patients with advanced cardiomyopathy, but no clear progression of the disease was observed, and no connection could be established among the variables studied and the variations in clinical status observed in advanced cases (mean time of observation, 11.8  $\pm$  2.4 months).

## DISCUSSION

In this longitudinal study, the results of xenodiagnosis showed a higher incidence of circulating parasites in Chagas' disease patients with cardiomyopathy (group 2) than in those without cardiomyopathy (group 1), as has also been reported by Maekelt (9). The serological tests demonstrated two relevant points. First, the titers were higher in group 2 than in group 1. Second, changes over time in antibody titers were more frequent in patients with a positive xenodiagnosis, demonstrating, as expected, an association between circulating parasites and humoral immune response. In group 1, patients with a positive xenodiagnosis had lower SI and NCPM in response to *T. cruzi* antigen than did those with a negative xenodiagnosis, whereas in group 2, reactivity to *T. cruzi* antigen was essentially equal between the xenodiagnostic subgroups.

In our opinion, these data indicate that, in patients with chagasic infection without any evidence of heart disease and a positive xenodiagnosis, a modulatory or regulatory mechanism that limits the intensity of the immune response to the antigen of the parasite is active, and this modulatory or regulatory mechanism is lost in patients with chagasic cardiomyopathy. This interpretation is in agreement with a report by A. B. Panagiotopoulos (Ph.D. thesis, Universidad Central de Venezuela, Caracas, 1984) showing that patients with chagasic infection without heart disease have a regulatory mechanism that is sensitive to indomethacin. Their monocytes, when incubated with *T. cruzi* antigen and indomethacin, have a higher SI than when incubated only with *T. cruzi* antigen. However, this regulatory mechanism is lost in patients with chagasic cardiomyopathy, as shown

by the similar SI and NCPM obtained in response to *T. cruzi* antigen either with or without indomethacin.

Recently it has been proposed that a cell-mediated autoimmune process against a heart antigen might be responsible for the development of chagasic cardiomyopathy. Santos-Buch and Texeira (15) have reported the presence of lymphocytes reactive to myocardial cells in rabbits experimentally infected with *T. cruzi*. More recently, Texeira et al. (18) have reported inhibition of leukocyte migration (ILM) in response to heart antigen in a small group of Chagas' disease patients. However, this interpretation can be questioned considering the small sample size and the findings of Wartenberg and Brostoff (22), who have shown that ILM may not be detecting a pathogenic autoreactivity to heart antigen, since ILM in response to heart antigen occurs in patients with coronary heart disease, and ILM has persisted for years after an episode of heart disease without causing any evidence of further pathology.

In our previous studies, we found that both patients with chagasic infection only and those with chagasic cardiomyopathy demonstrated positive cellular response to rat heart antigen (12). Our data clearly showed two points. First, the test was not positive in patients with other forms of heart disease, including coronary heart disease. Second, the frequency of patients reacting positively to rat heart antigen was practically the same in both groups (25 and 26.8%, respectively). These data did not, therefore, support a direct association between reactivity to heart antigen and chagasic cardiomyopathy. The data obtained in the present longitudinal study confirmed our previous findings of a similar frequency and intensity of positive lymphocyte proliferation in response to rat heart antigen in groups 1 and 2 (12 and 11%, respectively). Todd et al. recently have published similar data on different antigenic preparations (19). Of interest was the fact that they could show no significant response when human heart antigen was used, raising the possibility that the response to rat heart antigen observed by us, besides having no clear relationship with the presence of disease, might be heterophilic like EVI antibodies (6). The most important finding in our study was that no patient in either group had persistently positive reactivity to rat heart antigen, and no change in the clinical status of patients was associated with a positive response to this antigen.

Santos-Buch and Texeira (15) have postulated that lymphocyte autoreactivity is the consequence of the existence of an antigen that cross-reacts with myocardial cells and *T. cruzi*. If this was in fact the case, then the incidence of patients reacting positively to heart antigen should be higher among those with a positive xenodiagnosis. However, with our rat heart antigen preparation no relationship could be established between reactivity to rat heart antigen and a positive xenodiagnosis. These observations, together with the fact that reactivity to rat heart antigen was not persistent and had no evident relationship to the clinical status of patients, do not support the role of cell-mediated autoreactivity to heart antigen in the pathogenesis of chagasic cardiomyopathy.

In conclusion, the following fundamental points can be raised. The immune systems of Chagas' disease patients respond to *T. cruzi* antigen, but apparently this response is not sufficient to eliminate the parasite. The understanding of this failure will be of fundamental importance to the elucidation of the pathology of Chagas' disease.

The lower SI and NCPM in response to *T. cruzi* antigen in patients without heart disease and with a positive xenodiagnosis suggest that in patients with chagasic cardio-

myopathy a regulatory or modulatory mechanism is lost. The higher incidence of positive xenodiagnosis in group 2, possibly combined with a delayed hypersensitivity reaction to *T. cruzi*, associate the parasite with the pathology of heart tissue in Chagas' disease. The positive cellular responses to rat heart antigen detected are probably epiphenomena similar to the presence of autoantibodies or ILM in coronary heart disease (17, 22) and primary cardiomyopathy (7).

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